Influences of antibiotics on plantlet regeneration via organogenesis in loblolly pine (*Pinus taeda* L.)

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Abstract: Three antibiotics ampicillin, carbenicillin, and cefotaxime were evaluated for their effects on induction, growth, and differentiation of organogenic calli, as well as rooting of regenerated shoots of three loblolly pine (*Pinus taeda* L.) genotypes. Of the antibiotics administered, cefotaxime maximally increased the frequency of callus formation and growth rate of organogenic calli, carbenicillin maximally increased the frequency of shoot regeneration and the average number of adventitious shoots per piece of organogenic callus, ampicillin maximally decreased the rooting frequency of regenerated shoots and mean number of roots per regenerated shoot, in comparison with antibiotic-free media. Compared with the control, ampicillin minimally increased the frequency of callus formation, cefotaxime minimally increased the frequency of shoot regeneration, and carbenicillin minimally decreased the rooting frequency of regenerated shoots in three loblolly pine genotypes tested. All three antibiotics increased the frequencies of callus formation and shoot regeneration, and reduced the rooting frequency of regenerated shoots suggested that the establishment of an efficient *Agrobacterium tumefaciens*-mediated transformation protocol for stable integration of foreign genes into loblolly pine need to select a suitable antibiotic. This investigation could be useful for optimizing genetic transformation of conifers.

Key words: Antibiotic; *Pinus taeda* L.; Rooting ability; Shoot regeneration
Abbreviations: BA benzyladenine; 2,4-D 2,4-dichlorophenoxyacetic acid; IBA indole-3-butyric acid
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Introduction

Establishment of an efficient *Agrobacterium*-mediated transformation system depends not only the efficiency of the plantlet regeneration procedures but also on the subsequent elimination of this bacterium from transformed cells (Eapen 1990; Tang 2000). Genetic transformation in conifers mediated by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* vectors require the use of antibiotics in the selection and regeneration medium (Cheng *et al.* 1998; Hammerschlag *et al.* 1997; Mathias & Boyd 1986). Co-cultivated tissues need to be sub-cultured a number of times on the medium supplemented with antibiotics in order to control bacterial growth, without interfering with the regeneration potential of the transformed cells (Mathias &, Mukasa. 1987; Nakano and Mii 1993). Currently, ampicillin, carbenicillin, and cefotaxime are some of the most commonly used antibiotics for suppresssing *Agrobacterium* after co-cultivation of the plant tissues (Nauerby *et al.* 1997; Sarma 1995). The various positive effects of cefotaxime and carbenicillin on in vitro growth and shoot regeneration were documented well in *Antiyyhinum majus* (Holford & Newbury 1992), eggplant (Billings *et al.* 1997), and *Lycopersicon esculentum* (Ling *et al.* 1998), and reviewed (Nauerby *et al.* 1997; Teng & Nicholson 1997), but their possible effects on growth and differentiation of pine tissues have not been described. Although transgenic plants have been obtained from *Larix decidua* (Huang *et al.* 1991), *Larix kaempferi* × *L. decidua* (hybrid larch) (Levee *et al.* 1997), Norway spruce (*Picea abies* L.) (Wenck *et al.* 1999), and white pine (*Pinus strobus* L.) (Levee *et al.* 1999) via co-cultivation of explants with *Agrobacterium*, there is no report on the influences of antibiotics on in vitro regeneration of loblolly pine tissues derived from *Agrobacterium tumefaciens*-mediated transformation. Loblolly pine (*Pinus taeda* L.) is an economically important forest tree that is widely planted in tropical and subtropical regions. Since Sederoff *et al.* (1986) first reported the gene transfer into loblolly pine by *Agrobacterium tumefaciens*, extensive investigations have been conducted (Gupta et al. 1988; Humara *et al.*1999; Stomp *et al.* 1990; Wenck, *et al.* 1999). The aim of the work described here was to evaluate the influence of antibiotics incorporated in different media on the in vitro regeneration of three loblolly pine genotypes as a basis for future investigations on genetic transformation

Materials and methods

Plant materials and antibiotics

Mature seeds of loblolly pine (genotypes J-16, J-23, and Q-27) were collected from Zhangjiajie Loblolly Pine SeedOrchard, Hunan Province, China in October 1996, and stored in plastic bags at 4°C before they were used to tis-

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sue culture. Surface-sterilization was performed by immersion of the seeds in 70% (v/v) ethanol for 1 min, followed by 20 min in a 2.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween-20 and then four rinses in sterile distilled water. Thereafter seeds were soaked for 24 h at 25°C in sterile distilled water on a rotatory shaker (100 rpm). Seeds were germinated in 100mm × 15mm Petri dishes (Fisher Scientific Co., Suwanne, USA) with 15 ml germination medium containing TE salts [22], 300 mg/L Myo-inositol, 2 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/l nicotinic acid, 10 g/l sucrose, pH 5.8, and solidified with 0.8% agar (Sigma Co, St. Louis, USA). Cultures were maintained under a 16/8 h (light/dark) regime, 100 μmol $m^{-2} \cdot s \cdot ^{1}$ light radiation provided by four fluorescent tubes for three days. The temperature of the growth room was kept at 25°C. Antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt (all from Sigma Co, St. Louis, USA) were dissolved in distilled water. The filter sterilized solution of each antibiotics were respectively added to the autoclaved callus induction medium, callus growth medium, shoot regeneration medium, and rooting medium (Tang 1998) at the concentration of 500 mg/l, after the latter was autoclaved and cooled. Callus induction and growth were conducted in the dark, and shoot regeneration and rooting were conducted under a 16/8 h (light/dark) regime with 100 μmol $m^{-2} \cdot s \cdot ^{1}$ light radiation provided by fluorescent tubes.

Callus induction and growth

Mature zygotic embryos were aseptically removed from the megagametophytes and placed horizontally on a solidified germination medium in 125 mL Erlenmeyer flasks or 100 mm × 15 mm Petri dishes. Mature zygotic embryos were used as the source of cotyledonary explants for experiments after were cultured on germination medium for 1 week. Cotyledons were cut and were placed horizontally on a solidified callus induction medium consisted of TE medium (Tang 1998) supplemented with 10 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 4 mg/L benzyladenine (BA), 4 mg/L kinetin, and with or without (control) 500 mg/l antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt, respectively, for 6 weeks. All media were adjusted to pH 5.8 prior to autoclaving. These media were dispensed as 20 mL aliquots in disposable 15 mm × 100 mm plastic Petri dishes and sealed with Parafilm (Sigma Co. St. Louis, USA) after transfer of the explants. Experiments were performed with 10 explants per Petri dish. Each treatment was replicated three times, and each replicate consisted of 30 cotyledons. The frequency of callus formation was calculated as number of cotyledons forming callus divided by total number of cotyledons tested in the 6th week of callus induction. Then callus (0.5 cm × 0.5 cm in size, 0.5 g in weight) were transferred into callus growth medium consisted of TE medium (Tang 1998) supplemented with 4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/L benzyladenine (BA), 1 mg/L kinetin, and with or without (control) 500 mg/l antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt, respectively, for 6 weeks. Growth of calli was determined in the sixth of culture, respectively. Growth was evaluated by the increment (the increase in the growth mean number of transgenic calli) of the fresh weight of transgenic calli. Each treatment was replicated three times, and each replicate consisted of 30 pieces of callus tissues (0.5 cm × 0.5 cm in size, 0.5 g in weight). All media were adjusted to pH 5.8 prior to autoclaving. Tissue cultures were subcultured every three weeks.

Adventitious shoot regeneration and proliferation

Proliferated organogenic callus were transferred into shoot regeneration medium consisted of TE medium [22] supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l benzyladenine (BA), 0.5 mg/L kinetin, and with or without (control) 500 mg/l antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt, respectively, for 12 weeks. Both the frequency of organogenic calli forming adventitious shoots and the mean number of adventitious shoots per piece of organogenic calli (0.5 cm × 0.5 cm in size) was determined in the 12th of culture, respectively. The frequency of organogenic calli forming adventitious shoots was evaluated by the percentage of calli forming transgenic adventitious shoots in three genotypes (genotype J-16, J-23, and Q-27) tested. Each treatment was replicated three times, and each replicate consisted of 30 pieces of callus tissues (0.5 cm × 0.5 cm in size). All media were adjusted to pH 5.8 prior to autoclaving. Tissue cultures were subcultured every three weeks.

Rooting of regenerated shoots

Elongated shoots (1.5 – 3.0 cm) were individualized and transferred to rooting medium. Rooting medium consisted of TE medium (Tang 1998) supplemented with 0.1 mg/l IBA indole-3-butyric acid, 0.5 mg/L benzyladenine (BA), 0.1 mg/L kinetin, and with or without (control) 500 mg/l antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt, respectively, for 9 weeks. The frequency of rooting and the mean number of root per regenerated shoot were determined in the 9th of culture, respectively. The rooting response of shoots regenerated with or without antibiotics was compared. Experiments were performed with four shoots per 125 mL Erlenmeyer flask, with nine flasks per treatment. The culture conditions were the same as established for the regeneration phase. Tissue cultures were subcultured every three weeks.

Statistical analysis

Statistical analysis on data of the frequency of callus formation, callus growth rate, the frequency of shoot regeneration, mean number of regenerated shoots per gram callus, rooting frequency, and average number of roots per

regenerated shoot were performed with the analysis of variance (ANOVA), and mean comparison was made with the least significant difference test at 5% level of probability.

Results and discussion

Effects of the antibiotics on callus induction and growth

Calli were formed on cotyledons of three genotypes of loblolly pine on the 3-9th week of culture. The effect of antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt on the frequency of callus formation and callus growth rate were shown in Tables 1 and 2. In general, explants regenerated more callus on antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt -containing media than on those not supplemented with the antibiotic (Figure 1a – b). Compared to the control, callus induction frequencies were obviously improved. However, callus induction frequencies were not generally significant among three genotypes tested (Table 1). Analysis of the effects of antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt on the callus growth rate also showed that there was a positive influence on callus growth rate in all genotypes tested. Cefotaxime maximally increased both the frequency of callus formation and callus growth rate, ampicillin minimally increased the frequency of callus formation, and carbenicillin minimally increased the callus growth rate (Table 1 and 2), on callus induction medium and callus growth medium supplemented with 500 mg/l antibiotics (ampicillin, carbenicillin, cefotaxime, respectively).

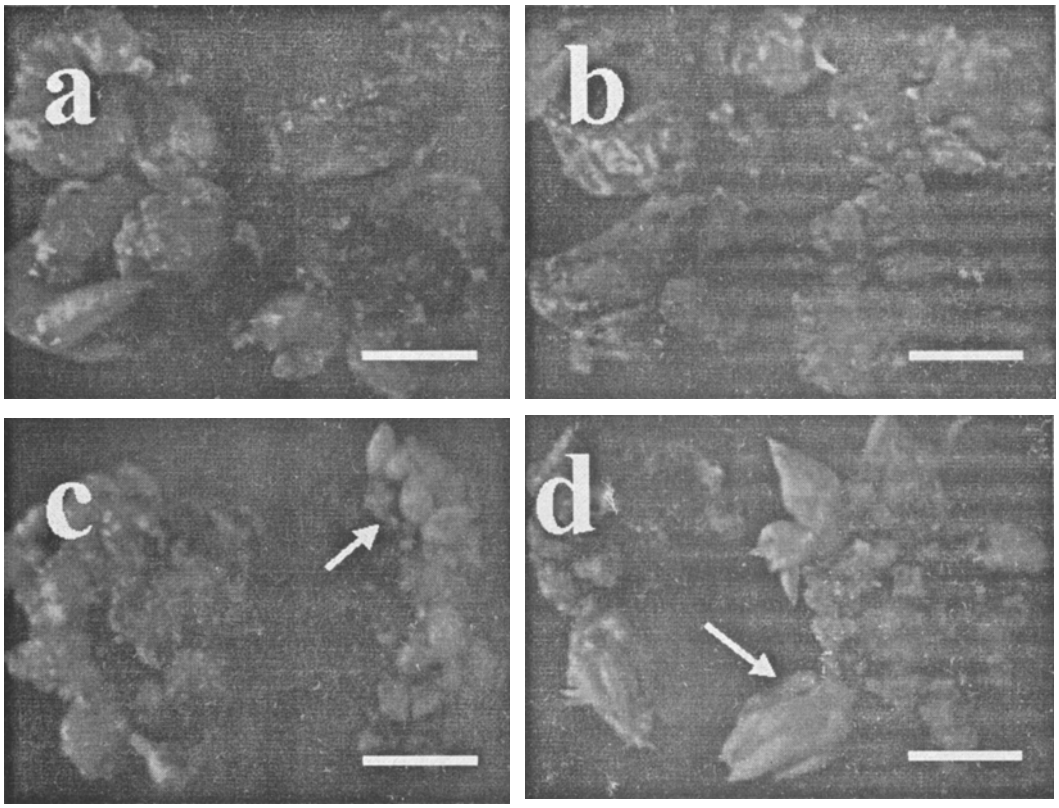

Fig. 1 Influence of antibiotics on growth and differentiation of organogenic callus in loblolly pine.
a Organogenic callus derived from cotyledons on callus induction medium without antibiotic carbenicillin (bar = 0.8 cm), b Organogenic callus derived from cotyledons on callus induction medium with antibiotic carbenicillin (bar = 0.8 cm), c Differentiation of organogenic callus and shoot regeneration on adventitious shoot regeneration medium without antibiotic carbenicillin (bar = 0.5 cm), d Differentiation of organogenic callus and shoot regeneration on adventitious shoot regeneration medium with antibiotic carbenicillin (bar = 0.5 cm) (Pictures are from genotype Q-27. Arrows indicate adventitious shoots)

Effects of the antibiotics on adventitious shoot regeneration

After organogenic callus were transferred to shoot regeneration medium, adventitious shoots were formed on the surface of callus (Figure 1c – d). Compared to the control, three genotypes of loblolly pine had obviously higher frequency of callus forming adventitious shoots and mean number of root per regenerated shoot on the regeneration medium with abtibiotics (Table 3 and 4). Among three antibiotics tested, carbenicillin maximally increased the frequency of shoot regeneration and mean number of regenerated shoots per pieces of callus, and cefotaxime minimally increased the frequency of shoot regeneration and mean number of regenerated shoots per pieces of callus (Table 3 and 4). On shoot regeneration medium with

(Table 3 and 4). On shoot regeneration medium with antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt, the frequencies of shoot regeneration were increased by 40.1%, 42.8%, and 35.7% (for genotype Q-27), respectively, (Table 3). Data on antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt on mean number of shoot regeneration per pieces of organogenic calli showed that mean number of adventitious shoots increased compared to the control. Ampicillin, carbenicillin, and cefotaxime resulted in a 48.8%, 67.4%, and 41.9% in mean number per piece of organogenic calli (for genotype Q-27) on the 12th week of culture (Table 4). Compared to cefotaxime, ampicillin and carbenicillin improved shoot regenration more.

Table 1. Effects of antibiotics on the percentage of cotyledon explants forming organogenic callus of three loblolly pine genotypes.

Antibiotics /500 mg·L^{-1}	Genotypes of loblolly pine J-16	J-23	Q27
Ampicillin	31.1 ± 2.8a	32.4 ± 3.1a	33.1 ± 2.7a
Carbenicillin	32.7 ± 3.4a	33.1 ± 2.9a	34.3 ± 3.5a
Cefotaxime	33.6 ± 3.9a	33.7 ± 3.5a	35.7 ± 3.9a
Control	22.9 ± 1.3b	23.6 ± 2.7b	25.3 ± 2.8b

Each treatment was replicated three times, and each replicate consisted of 30 cotyledon explants (0.1 cm × 0.2 cm in size). Percentage of callus formation were determined by the number of cotyledons forming organogenic callus/total number of explants. Means followed by the same letter are not statistically different at $P \leq 0.05$.

Table 2. Effects of antibiotics on the growth rate (mg/d/g) of organogenic callus of three loblolly pine genotypes.

Antibiotics /500 mg·L^{-1}	Genotypes of loblolly pine J-16	J-23	Q27
Ampicillin	14.1 ± 2.3a	15.1 ± 2.7a	17.1 ± 2.4a
Carbenicillin	13.7 ± 3.5a	14.5 ± 2.3a	16.3 ± 2.5a
Cefotaxime	14.6 ± 3.9a	15.7 ± 3.6a	17.7 ± 3.3a
Control	10.9 ± 1.3b	11.6 ± 2.1b	12.2 ± 2.8b

Each treatment was replicated three times, and each replicate consisted of 30 pieces of organogenic callus tissues (0.5 gram per piece). Growth rate of organogenic callus were determined by the increasing weight (mg) of callus /day/gram initial callus in six weeks. Means followed by the same letter are not statistically different at $P \leq 0.05$.

Table 3. Effects of antibiotics on the percentage (%) of organogenic callus forming adventitious shoots of three loblolly pine genotypes.

Antibiotics /500 mg·L^{-1}	Genotypes of loblolly pine J-16	J-23	Q27
Ampicillin	33.1 ± 2.4a	34.1 ± 2.8a	41.6 ± 3.1a
Carbenicillin	35.3 ± 3.7a	36.9 ± 2.5a	42.1 ± 2.7a
Cefotaxime	31.7 ± 3.5a	33.7 ± 3.4a	40.3 ± 3.5a
Control	22.9 ± 1.3b	23.6 ± 2.1b	29.7 ± 2.8b

Each treatment was replicated three times, and each replicate consisted of 30 pieces of organogenic callus tissues (0.5 cm × 0.5 cm in size). Percentage of organogenic callus forming adventitious shoots were determined by the number of organigenic callus forming adventitious shoots/total number of organogenic callus. Means followed by the same letter are not statistically different at $P \leq 0.05$.

Table 4. Effects of antibiotics on the mean number of adventitious shoots per piece of organogenic callus of three loblolly pine genotypes.

Antibiotics (500 mg·L^{-1})	Genotypes of loblolly pine J-16	J-23	Q27
Ampicillin	5.3 ± 1.4a	5.4 ± 1.3a	6.4 ± 1.2a
Carbenicillin	5.8 ± 1.5a	6.1 ± 1.2a	7.2 ± 1.1a
Cefotaxime	5.1 ± 1.2a	5.2 ± 1.5a	6.1 ± 1.0a
Control	3.7 ± 1.3b	3.9 ± 1.1b	4.3 ± 1.8b

Each treatment was replicated three times, and each replicate consisted of 30 pieces of organogenic callus tissues (0.5 cm × 0.5 cm in size). The mean number of adventitious shoots per piece of organogenic callus was determined in the 12th week of culture. Means followed by the same letter are not statistically different at $P \leq 0.05$.

Effects of the antibiotics on rooting ability

The data on antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt on rooting ability of regenerated shoots in loblolly pine presented here demonstrated that the rooting frequency and average number of roots per regenerated shoot was decreased by antibiotics (Figure 2 and 3). Compared to the control, carbenicillin minimally decreased the rooting frequency and the mean number of roots per regenerated shoot, and ampicillin maximally decreased the rooting frequency and the mean number of roots per regenerated shoot in loblolly pine (Figure 2 and 3).

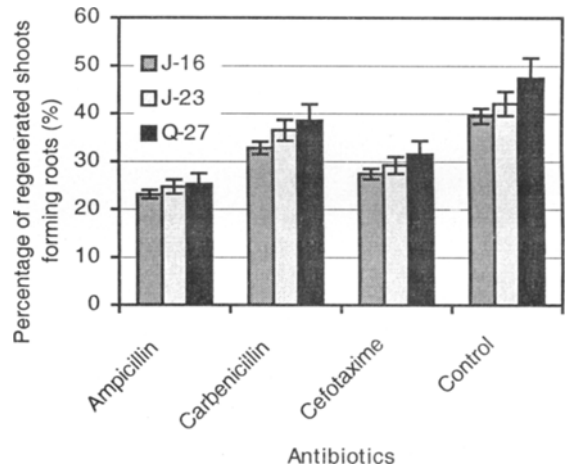

Fig. 2 Influence of different antibiotics ampicillin, carbenicillin, and cefotaxime (500 mg/l each) on rooting ability of regenerated shoots of three loblolly pine genotypes. Each treatment was replicated three times, and each replicate consisted of 36 regenerated shoots (2 – 3 cm in height). Errors represent the standard deviation around the mean.

Ampicillin, carbenicillin, and cefotaxime possess auxin-like structural features, and when broken down display effects similar to those of the weak auxin

play effects similar to those of the weak auxin phenylacetic acid in the culture medium, thereby, increasing the regeneration potential of cultured explants (Lin *et al.* 1995, Ling *et al.* 1998). The enhancement of the regeneration potential of cultured explants by carbenicillin and penicillin G had been reported (Lin *et al.* 1995; Robert *et al.* 1989). Therefore, the enhancement in growth and differentiation of organogenic calli observed in the present work using antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt may be a result of the possible release of auxin-like compounds, as has been reported for carbenicillin and penicillin G (Holford and Newbury 1992; Lin *et al.* 1995; Robert *et al.* 1989). For other species the various positive effects of antibiotics like cefotaxime and carbenicillin on shoot regeneration are well-documented and reviewed (Billings *et al.* 1997; Holford and Newbury 1992; Nauerby *et al.* 1997).

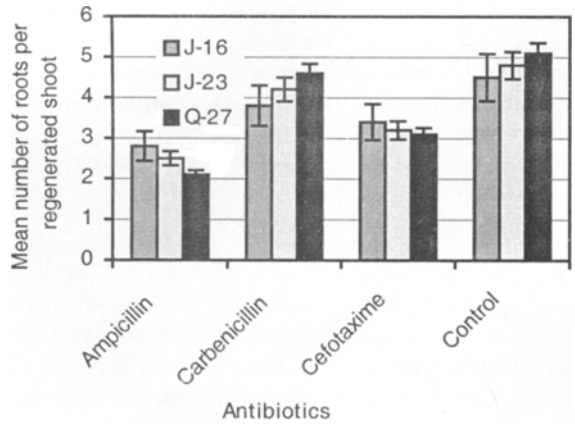

Fig. 3 Influence of different antibiotics ampicillin, carbenicillin, and cefotaxime (500 mg/l each) on mean number of roots per regenerated shoots of three loblolly pine genotypes. Each treatment was replicated three times, and each replicate consisted of 36 regenerated shoots (2 – 3 cm in height). Errors represent the standard deviation around the mean.

Nauerby *et al.* (1997) investigated the influence of timentin (150 mg/l) on the regeneration potential of *Nicotiana tabacum* 'Petit Havana' SR1 leaf discs and cotyledon explants and compared these with the effects of cefotaxime (1500 mg/l) or carbenicillin (1000 mg/l). A positive influence of timentin was found on shoot regeneration from leaf discs (127% after a 1-month culture). However, no influence on shoot production or rooting ability from cotyledons was observed after 1 month. Ling *et al.* (1998), in experiments involving the *Agrobacterium*-mediated transformation of tomato cultivar 'Moneymaker', reported positive effects of ticarcillin/potassium clavulanate (150 mg/l) on callus growth and shoot regeneration. According to those authors, transformation frequencies on the ticarcillin/ potassium clavulanate-containing medium were about 40–50% higher than on the cefotaxime-containing medium, and four to five fold more transformed plants were obtained using ticarcillin/potassium clavulanate.

Our results suggest that antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt play an important role in callus induction, growth, and shoot regeneration in loblolly pine. These results corroborate with other findings related to the positive influence of antibiotics on callus growth and shoot regeneration (Robert *et al.*1989; Teng & Nicholson 1997). Although, there was a significant increase in the frequencies of shoot regeneration from organogenic calli cultured on shoot regeneration medium with antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt (Table 3), a decrease in both rooting frequency and average number of roots per regenerated shoot (Figure 2 and 3) was observed in those shoots, compared to the control. Negative effects of antibiotics added to the rooting medium on rooting have also been observed (Costa *et al.* 2000; Nauerby *et al.* 1997). Nauerby *et al.* (1997) suggested that the negative effects on rooting associated with carbenicillin and cefotaxime were transmitted from the regeneration medium, via the regenerated shoots, indicating a rather strong and long-lasting effect of the components from the regeneration medium. Together with our data, these results suggest the positive and long-lasting effect may be species- and/or cultivar-dependent. The present results from callus induction and shoot regeneration on media supplemented with antibiotics confirm those found in previous reports regarding positive effects associated with the incorporation of antibiotics in the culture medium and their positive effects upon shoot regeneration in vitro (Holford & Newbury. 1992; Lin *et. al.* 1995). In short, the results described here document the effects of antibiotics ampicillin sodium salt, carbenicillin disodium salt, and cefotaxime sodium salt on callus induction, shoot regeneration, and rooting of regenerated shoots in loblolly pine. In contrast to ampicillin and cefotaxime, carbenicillin is more suitable for

Selecting as a antibiotics in transformation experiments in loblolly pine genotypes tested, because carbenicillin maximally increased the frequency of shoot regeneration and minimally decreased the rooting frequency in three loblolly pine genotypes tested in this investigation.

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